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In vitro culture of arabidopsis, regeneration and cell suspension

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Abstract
An understanding of basic methods in Arabidopsis tissue culture is beneficial for any laboratory working on this model plant. Tissue culture refers to the aseptic growth of cells, organs, or plants in a controlled environment, in which physical, nutrient, and hormonal conditions can all be easily manipulated and monitored. The methodology facilitates the production of a large number of plants that are genetically identical over a relatively short growth period. Techniques, including callus production, cell suspension cultures, and plant regeneration, are all indispensable tools for the study of cellular biochemical and molecular processes. Plant regeneration is a key technology for successful stable plant transformation, while cell suspension cultures can be exploited for metabolite profiling and mining. In this chapter we report methods for the successful and highly efficient *in vitro* regeneration of plants and production of stable cell suspension lines from leaf explants of *Arabidopsis thaliana.*

Keywords: Callus, cell suspensions, plant regeneration, tissue culture, arabidopsis, organ regeneration

1. Introduction
Plant tissue culture is an indispensable tool for the study of cellular biochemical and molecular processes and a key technology for successful stable plant transformation. *In vitro* culture, from the Latin "in glass," was so named for the glass vessels that the cultures were grown in and is a term which probably came into use at the end of the nineteenth century by embryologists. The earliest attempts at tissue culture of plant cells were made in the first decade of the 1900s by the Austrian Botanist Haberlandt who published his work in German (translated into English in ref. 1). However, it wasn't until 30 years later, following the discovery of plant growth regulators, that the development of the technique to include auxins allowed for the possibility of cultivating plant tissue in an aseptic environment for an indefinite length of time [2–4]. Further advancements in nutrient and micronutrient content, plant growth regulator (PGR) discovery, and manipulation of ratios of PGR have all dramatically improved the efficiency and versatility of the technique to bring us to where we are today with the ability to cultivate callus, cell suspensions, protoplasts, organs, and regenerate whole plants. Culturing techniques provide a tightly controlled closed growth system while facilitating the manipulation of experimental conditions. Physical, nutritional, and hormonal states can all be easily regulated in the closed system reducing variability and extraneous factors. The generation of plant material in this manner offers a homogenous and genetically identical pool which, through the process of subculturing, can result in large quantities of experimental material over very short time frames, while the sterile growth conditions ensure the material is free from pathogenic microorganisms.
In vitro culture to produce cell suspensions or regenerate plants
begins with the selection of explant material. The explant is a highly differentiated piece of tissue (i.e., leaf pieces) harvested from the plant, that is sterilized and placed on an artificial nutrient and vitamin-rich, PGR-supplemented, growth medium. The wounding of the tissue and the presence of specific amounts of PGR induce somatic embryogenesis, and the cells in the media begin to revert to their meristematic state, dividing rapidly and dedifferentiating to form a mass of unorganized cells called callus. These differentiated cells can either be maintained indefinitely as callus through subculturing to prevent nutrient deficiency or, once the cells of the callus become less packed and more friable, be transferred to liquid medium where they dissociate into single cells to generate stable cell suspension cultures.

Undifferentiated callus or suspension cells can be stimulated by PGR to initiate organogenesis exploiting the ability of all plant cells, due to their genetic potential, to dedifferentiate and then, under defined conditions, to redifferentiate to form any plant organ, a phenomenon known as totipotency [5]. Particularly important in organogenesis is the ratio of the PGRs' cytokinin and auxin. Typically, a high ratio of cytokinin to auxin results in shoot differentiation, whereas a high ratio of auxin to cytokinin induces differentiation of cells to roots [6]. However, there are plants that provide exceptions to this rule.

The first reports of Arabidopsis callus culture date back to the 1960s [7]. These were followed by articles detailing methods for cell suspensions as well as organ and plant regeneration [8, 9]. This early methodology was applied nearly a decade later to the regeneration of whole plants from *Agrobacterium tumefaciens- transformed* Arabidopsis leaf explants [10]. Early contributions to the field such as these led to the Arabidopsis molecular revolution that continues to this date and has established this small, unassuming weed as an unrivalled model plant system [11].

Here we report methods for the efficient and successful regeneration of plants and production of stable cell suspension lines from dedifferentiated callus produced from leaf explants of *Arabidopsis thaliana.*

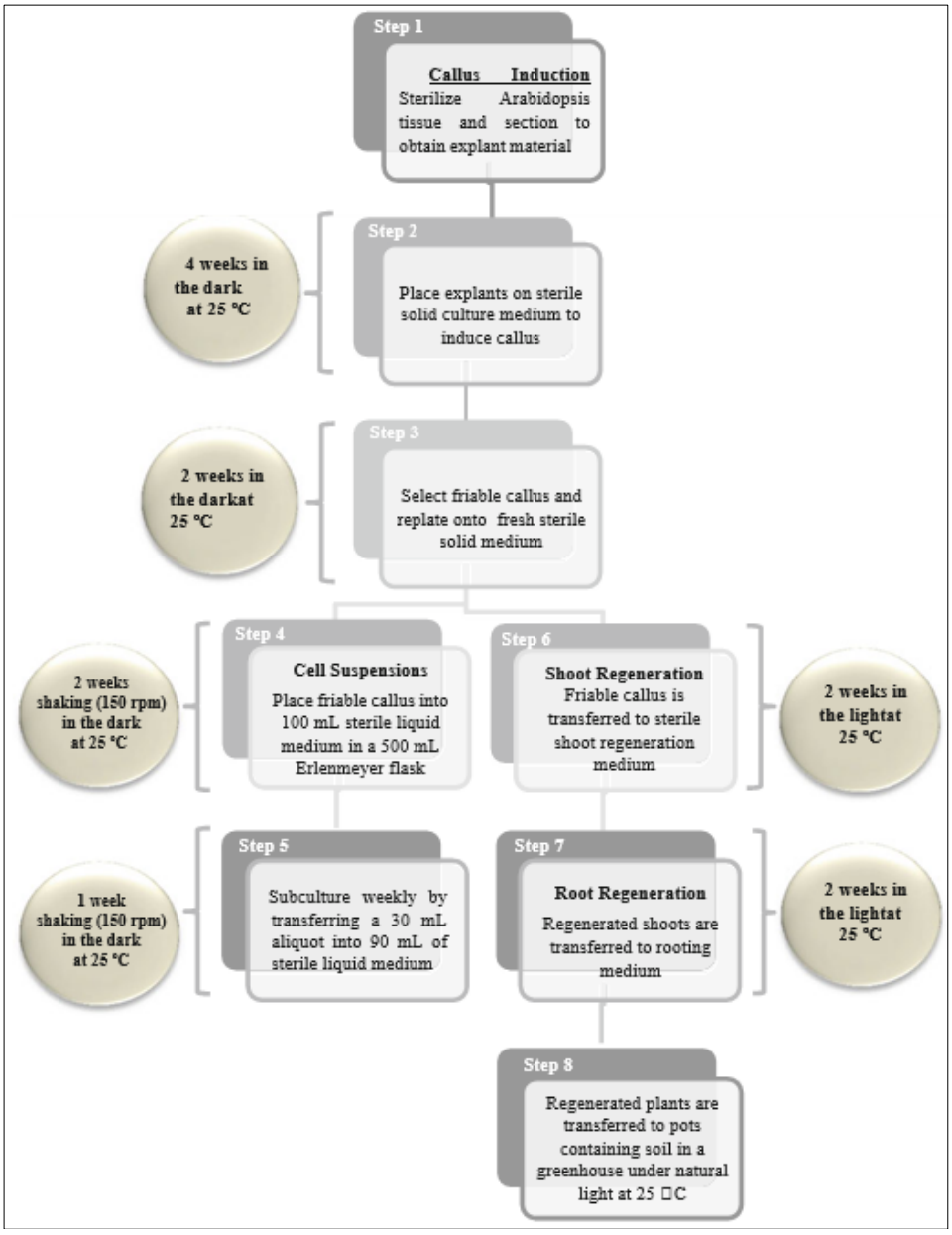

Fig 1: Schematic diagram of the steps and time involved in the production of callus cultures, cells suspension cultures, and regenerated plants from Arabidopsis

2. Materials

1. Dry seeds of *A. halleri* and *A. thaliana*.
2. 100 mm diameter pots.
3. MetroMix 510 soilless mixture combined with perlite (3:1).
4. 90 % (v/v) ethanol.
5. 70 % (v/v) ethanol.
6. 5 % sodium hypochlorite solutions (bleach).
7. Sterilized deionized water.
8. Microcentrifuge.
9. Petri plates 9 cm.
10. Magenta tissue culture boxes.
11. Pair of forceps.
12. Scalpel and sterile scalpel blades.

13. Bunsen burner.
14. Gamborg's B5 vitamins [12]: 10 mg/L thiamine hydrochloride, mg/L nicotinic acid, 1 mg/L pyridoxine hydrochloride, 100 mg/L myoinositol. Prepare as a 100× stock solution in ster- ile deionized water, and add to the medium before autoclaving. The stock can be stored at 4 °C.
15. Basic Murashige and Skoog medium [13] (*see* Table 1 and Notes 1–3). Stock solutions (1–6) are prepared in a total volume of 100 mL.
16. Medium 1 (M1): basic MS medium, supplemented with Gamborg's B5 vitamins, 3 % sucrose, 1.5 % bacteriological agar, 1 mg/L 2,4-D, and 0.05 mg/L benzylaminopurine (BA) (*see* Note 4). Adjust to pH 5.7 with 1 N KOH.
17. Shoot regeneration medium, M2: basic MS medium supple- mented with Gamborg's B5 vitamins, 3 % sucrose, 1.5 % bacteriological agar, 0.5 mg/L 2,4-D, and 0.1 mg/L BA.
18. Root regeneration medium, M3: half-strength MS medium supplemented with half-strength Gamborg's B5 vitamins (5 mg/L thiamine hydrochloride, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine hydrochloride, 50 mg/L myoinositol), 1 % sucrose, 1 % bacteriological agar, and 0.3 mg/L 2,4-D. Adjust the pH to 5.7 with 1 N KOH.
19. Hoagland and Arnon [14] hydroponic stock solutions: 1 M $(NH_4)_2HPO_4$ (add 1 mL/L), 1 M KNO_3 (add 6 mL/L), 1 M $Ca(NO_3)_2{\cdot}4H_2O$ (add 4 mL/L), $MgSO_4{\cdot}7H_2O$ (2 mL/L), 1 mL/L of micronutrients stock (2.85 g/L H_3BO_3, 2.44 g/L $MnCl_2{\cdot}4H_2O$, 0.22 g/L $ZnSO_4{\cdot}7H_2O$, 0.08 g/L $CuSO_4{\cdot}5H_2O$, 0.02 g/L $H_2MoO_4{\cdot}H_2O$), 10 mL/L of Fe2+ stock solution (0.4129 g/L $Na_2EDTA{\cdot}2H_2O$ and 0.278 g/L $FeSO_4{\cdot}7H_2O$) (*see* Note 5).
20. Suspension culture medium (S1). This medium is the same as M1 but does not contain agar.
21. Sterile 500 mL Erlenmeyer flasks with cotton plugs.
22. Pipette pump and sterile 10 mL pipettes.
23. 1 mL micropipette and sterile tips.
24. Aluminum foil.
25. Parafilm.
26. Transparent plastic cups.
27. Rotatory shaker set at 150 rpm with temperature control.
28. Growth chamber set at 25 °C.
29. Balance.
30. Microscope and microscope slides.
31. Filtration unit and sterile filters (0.22 μm).
32. Oven.
33. Autoclave (120 °C, 20 min).
34. Laminar flow hood/cabinet.
35. Neutral red stock solution (4 mg/ml in deionized water).

Table 1: Murashige and Skoog stock solutions and amounts added to prepare 1 L of basic MS media

MS medium stocks (*see* Note 3)	Chemicals	g/100 mL stock	ml/L media
Stock solution 1	NH_4NO_3	16.5	10
	KNO_3	19.0	
	$MgSO_4{\cdot}7H_2O$	3.7	
	KH_2PO_4	1.7	
Stock solution 2	$CaCl_2$	4.4	10
Stock solution 3	$NaEDTA{\cdot}2H_2O$	0.37	10
	$FeSO_4{\cdot}7H_2O$	0.28	
Stock solution 4	H_3BO_3	0.062	10
	$MnSO_4{\cdot}H_2O$	0.169	
	$ZnSO_4$	0.086	
Stock solution 5	KI	0.083	1.0
	$Na_2MoO_4{\cdot}7H_2O$	0.025	
Stock solution 6	$CuSO_4{\cdot}5H_2O$	0.025	0.1
	$CoCl_2{\cdot}6H_2O$	0.025	

3. Methods

3.1 Working in a Sterile Transfer Hood

1. A sterile working environment is critical to prevent contamination of the plant tissue and medium by microorganisms (bacteria and fungi). If contamination does occur these microorganisms will rapidly colonize the media due to the high sugar and nutrient con- tent and destroy the plant material.
2. Clean all surfaces inside sterile laminar hood/cabinet with 70 % ethanol and allow to air dry. Make sure there are no Bunsen burners on during this step.
3. Place only the necessary material and tools inside the hood/ box and remove the material when it is no longer needed. All material should be sterile and tools should be clean and prefer- ably wiped with a solution of 70 % alcohol.
4. Work with your arms extended into the hood/cabinet and your head and body outside. Try and use only the back 1/3 of the hood as this is the most sterile area; do not obstruct the HEPA air filter with material or body parts as this will affect the laminar air flow and may result in contamination.

3.2 Method for Callus Production

1. Seeds are propagated in 100 mm diameter pots in MetroMix 510 combined with perlite (3:1) for 4 weeks (*A. thaliana*) at which time they are used in the establishment of axenic cell culture as follows.
2. Leaves are washed by immersion in 90 % (v/v) ethanol in a sterile Petri plate for 1 min followed by three rinses with sterile deionized water.
3. Leaves are then surface sterilized by immersion in a 5 % sodium hypochlorite solution and incubated for 10 min with gently mixing.
4. Remove the sodium hypochlorite using a sterile 1 mL pipette tip and rinse the leaves five times with sterile deionized water.

5. After elimination of the water, the sterile leaves are sectioned into 2–4 small pieces using a sterile scalpel blade in a sterile Petri plate.
6. The abaxial sides of the sterile sectioned leaves are placed onto media M1, and Petri plates are sealed with parafilm, covered with aluminum foil and incubated at 25 °C.
7. Following a period of 4 weeks friable calli are obtained (*see* Fig. 2a, b and Note 6).

3.3 Method for Regeneration of Plants

1. Friable callus (0.5 g) obtained as indicated in Subheading 3.2 is transferred aseptically to shoot regeneration medium M2, in 9 cm Petri plates, and incubated under a 16-h day length with a photon flux density of 350 µmol m2/s at 25 °C for 4 weeks.
2. Callus is monitored biweekly for shoot generation.
3. Once shoots containing 2–3 leaf pairs have developed on the calli (see Fig. 2b), these are selected and transferred with the callus to a root regeneration medium M3, in deep transparent sterile containers such as Magenta boxes (see Note 7), and placed in a growth room under a 16-h day length with a photon flux density of 350 µmol/m/s at 25 °C.
4. Root organogenesis is monitored until an abundant root system is formed and the elongated shoots are approximately 2 cm tall (see Fig. 2c). This takes an additional 4 weeks.
5. Plants can be transferred to either dark hydroponic containers (to avoid algae growth) containing 0.5× Hoagland and Arnon solution or into soil in 100 mm pots, under natural light and humidity conditions in a greenhouse maintained at 25 °C (see Fig. 2d and Notes 8 and 9).

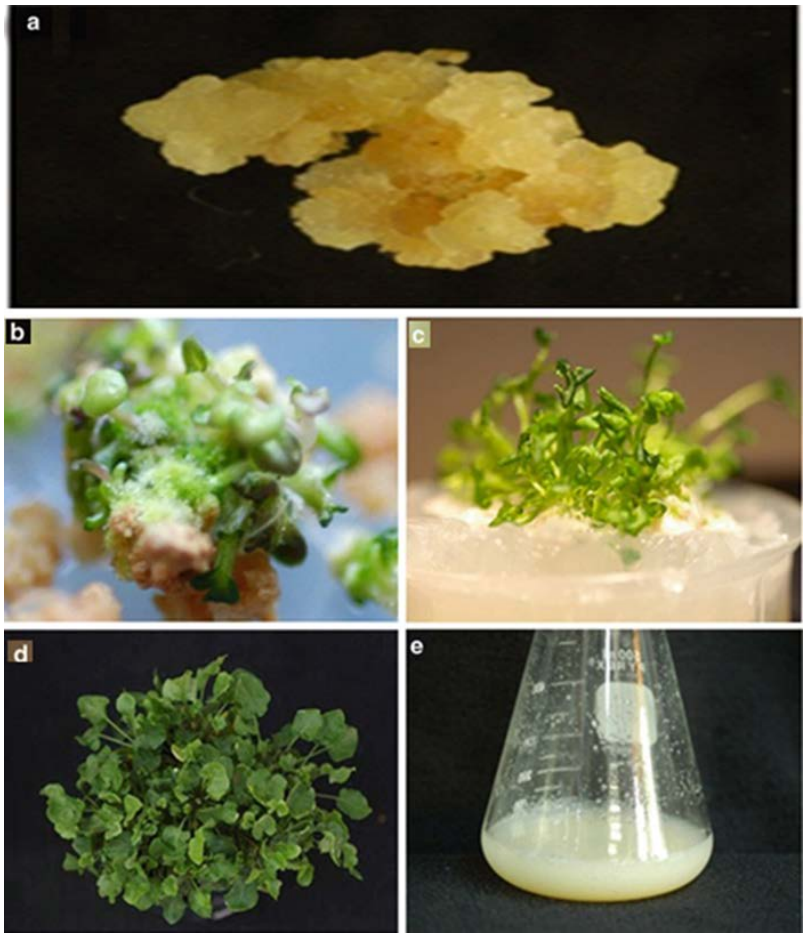

Fig 2: *In vitro* culture of Arabidopsis. Callus tissue is generated from leaf explants of (a) and *Arabidopsis thaliana* (a). Once friable callus is produced it is transferred aseptically to shoot regeneration media (b), followed by root regeneration media (c). The final step is the removal of the fully regenerated plants to the greenhouse (d). Friable callus can also be used to generate stable cell suspension cultures (e)

3.4 Method for Establishment of Cell Suspension Cultures

1. Transfer aseptically the friable calli (0.5 g) obtained as indi- cated in Subheading 3.2 into 100 mL of sterile S1 medium in a 500 mL Erlenmeyer flask and swirl the flask to break up the callus tissue into small pieces.
2. Place the flask on a shaker with continuous shaking (150 rpm), in the dark at 25 °C (*see* Note 10).
3. After a 2-week period, transfer aseptically 10 mL of cells into a sterile 500 mL Erlenmeyer flask containing 90 mL fresh S1 medium by using a 10 mL sterilized glass pipette connected to a pipette pump. Place the flask back onto the shaker (*see* Note 11).
4. Subculture the cell suspensions every 7 days to maintain the cells in the log phase of growth (*see* Fig. 2e and Note 12).

4. Notes

1. It is more economical and allows for easier manipulation of nutrients if MS medium is made from scratch as described in Table 1. However, it can also be purchased from several sources in pre-weighed packets.

2. $CaCl_2$ will precipitate if added to stock 1. Therefore, make it as an individual stock as indicated in Table 1.
3. All MS media solutions are stored at 4 °C with the exception of solution 1 which is maintained at room temperature to prevent solidification at the colder temperature.
4. Stocks of growth regulators are prepared at a 1 mg/mL concentration in sterile deionized water and added before autoclaving. These stocks are stored at 4 °C.
5. Hoagland's solutions are sterilized for 20 min at 120 °C with the exception of the $Ca(NO_3)_2$ and Fe^{2+} solutions that must be sterilized by filtration. In addition the Fe^{2+} solution must be heated before filter sterilization to oxidize the ferrous.
6. Calli are considered friable when the cells separate easily from the mass and are no longer dense and compacted.
7. Magenta tissue culture boxes are commonly used, but economic replacements are glass baby food jars with lids that can be sterilized.
8. To avoid rapid dehydration and plant stress, newly transferred plantlets need to acclimatize to lower humidity levels and should therefore be covered with small transparent plastic cups to maintain adequate humidity. Small holes can be punched into the covers to gradually decrease the humidity over a period of 1 week to that of the atmosphere. This ensures a 100 % survival rate of the regenerated plants.
9. Arabidopsis plants grown in hydroponics do not require aeration of the roots.
10. It is important to remove a small aliquot of cells from the culture to visualize under a microscope and check for cell viability every 2 days. Cell viability can be observed using a drop of neutral red dye. Cells which are viable will accumulate the dye.
11. The top of the Erlenmeyer flask containing the fresh media should be flame sterilized after removing the plug to create an upward hot air draft which directs particles away from the opening. This is repeated after adding the 10 mL of cells before replacing the plug.
12. To culture the cell suspensions for more than 8 days results in rapid browning and cell death as the availability of nutrients diminishes with culturing time. It is recommended to perform a growth curve to determine the cell culture doubling time. It is important to consider that the cell suspension growth varies in each species or cultivar.

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